

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(1) International Patent Classification 5 : C12N 15/44, 15/86, A61K 39/145 A1 (11) International Publication Number: WO 93/19183 (43) International Publication Date: 30 September 1993 (30.09.93)

(21) International Application Number: PCT/US93/02394  
(22) International Filing Date: 17 March 1993 (17.03.93)

(30) Priority data:  
855,562 23 March 1992 (23.03.92) US  
009,833 27 January 1993 (27.01.93) US

(71) Applicant: UNIVERSITY OF MASSACHUSETTS MEDICAL CENTER [US/US]; 55 Lake Avenue North, Worcester, MA 01655 (US).

(72) Inventors: ROBINSON, Harriet, L. ; 3 Birchwood Drive, Southboro, MA 01772 (US). FYNAN, Ellen, F. ; 13 Redstone Place, Sterling, MA 01564 (US). WEBSTER, Robert, G. ; 295 Richbriar Street, Memphis, TN 38120 (US).

(74) Agents: GRANAHAH, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

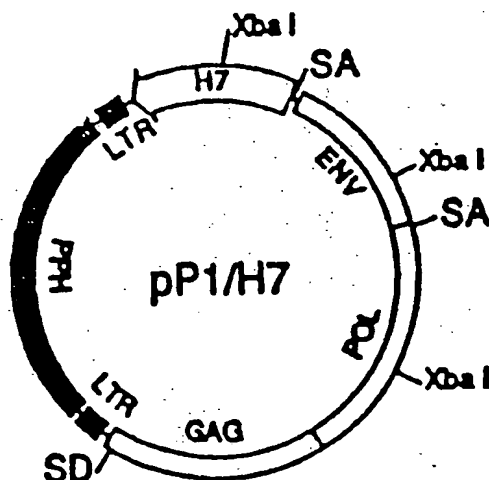
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

*With international search report.*

*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: IMMUNIZATION BY INOCULATION OF DNA TRANSCRIPTION UNIT



(57) Abstract

This invention relates to a method of immunizing a vertebrate, comprising introducing into the vertebrate a DNA transcription unit which comprises DNA encoding a desired antigen or antigens. The uptake of the DNA transcription unit by a host vertebrate results in the expression of the desired antigen or antigens, thereby eliciting humoral or cell-mediated immune responses or both humoral and cell-mediated responses. The elicited humoral and cell-mediated immune response can provide protection against infection by pathogenic agents, provide an anti-tumor response, or provide contraception. The host can be any vertebrate, avian or mammal, including humans.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                  | FR | France                                | MR | Mauritania               |
| AU | Australia                | GA | Gabon                                 | MW | Malawi                   |
| BB | Barbados                 | GB | United Kingdom                        | NL | Netherlands              |
| BE | Belgium                  | GN | Guinea                                | NO | Norway                   |
| BF | Burkina Faso             | GR | Greece                                | NZ | New Zealand              |
| BG | Bulgaria                 | HU | Hungary                               | PL | Poland                   |
| BJ | Benin                    | IE | Ireland                               | PT | Portugal                 |
| BR | Brazil                   | IT | Italy                                 | RO | Romania                  |
| CA | Canada                   | JP | Japan                                 | RU | Russian Federation       |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan                    |
| CG | Congo                    | KR | Republic of Korea                     | SE | Sweden                   |
| CH | Switzerland              | KZ | Kazakhstan                            | SK | Slovak Republic          |
| CI | Côte d'Ivoire            | LJ | Liechtenstein                         | SN | Senegal                  |
| CM | Cameroon                 | LK | Sri Lanka                             | SU | Soviet Union             |
| CS | Czechoslovakia           | LU | Luxembourg                            | TD | Chad                     |
| CZ | Czech Republic           | MC | Monaco                                | TC | Togo                     |
| DE | Germany                  | MG | Madagascar                            | UA | Ukraine                  |
| DK | Denmark                  | ML | Mali                                  | US | United States of America |
| ES | Spain                    | MN | Mongolia                              | VN | Viet Nam                 |
| FI | Finland                  |    |                                       |    |                          |

-1-

IMMUNIZATION BY INOCULATION OF DNA  
TRANSCRIPTION UNIT

Background of the Invention

Vaccination with inactivated or attenuated organisms  
5 or their products has been shown to be an effective method  
for increasing host resistance and ultimately has led to  
the eradication of certain common and serious infectious  
diseases. The use of vaccines is based on the stimulation  
of specific immune response within a host or the transfer  
10 of preformed antibodies. The prevention of certain  
diseases, such as poliomyelitis, by vaccines represents  
one of immunologies greatest triumphs.

Effective vaccines have been developed for relatively  
few of the infectious agents that cause disease in  
15 domestic animals and man. This reflects technical  
problems associated with the growth and attenuation of  
virulent strains of pathogens. Recently effort has been  
placed on the development of subunit vaccines (vaccines  
that present only selected antigens from a pathogen to the  
20 host). Subunit vaccines have the potential for achieving  
high levels of protection in the virtual absence of side  
effects. Subunit vaccines also offer the opportunity for  
the development of vaccines that are stable, easy to  
administer, and sufficiently cost-effective for widespread  
25 distribution.

Summary of the Invention

This invention relates to a method of immunizing an  
individual, comprising introducing into the individual a  
DNA transcription unit which comprises DNA encoding a  
30 desired antigen or antigens. The uptake of the DNA  
transcription unit by host cells results in the expression  
of the desired antigen or antigens, thereby eliciting

-2-

humoral or cell-mediated immune responses or both humoral and cell-mediated responses. The elicited humoral and cell-mediated immune response can provide protection against infection by pathogenic agents, provide an anti-tumor response, or provide contraception. The host can be any vertebrate, avian or mammal, including humans.

The present invention relates in a particular embodiment to a method of immunizing an individual by contacting a mucosal surface in the individual with a DNA transcription unit capable of expressing a desired antigen or antigen.

The DNA transcription unit introduced by the present method can be used to express any antigen encoded by an infectious agent, such as a virus, a bacterium, a fungus, or a parasite, as well as antigenic fragments and peptides that have been experimentally determined to be effective in immunizing an individual against infection by a pathogenic agent. As stated above, DNA transcription units can also be used for contraceptive purposes or for anti-cancer therapy.

The desired antigen to be expressed can be designed so as to give internal, surface, secreted, or budding and assembled forms of the antigens being used as immunogens.

There are numerous advantages for the use of DNA for immunizations. For example, immunization can be accomplished for any antigen encoded by DNA. Furthermore, the DNA encoded antigens are expressed as "pure" antigens in their native states and have undergone normal host cell modifications. Also, DNA is easily and inexpensively manipulated and is stable as a dry product or in solution over a wide range of temperatures. Thus, this technology is valuable for the development of highly effective subunit vaccines.

-3-

Brief Description of the Drawings

Figure 1 is an illustration of a bacterial plasmid containing a DNA transcription unit (referred to as pP1/H7) comprising an influenza virus hemagglutinin type 7 (H7) gene expressed by a replication competent retroviral vector.

Figure 2 is an illustration of a bacterial plasmid containing a DNA transcription unit (p188) comprising an influenza virus hemagglutinin type 7 (H7) gene expressed by a replication defective retroviral vector.

Figure 3 is an illustration of a bacterial plasmid comprising a retroviral vector (pRCAS) with no H7 insert, used as a control.

Figure 4A is a schematic representation of the nonretroviral vector comprising the influenza virus antigen DNA transcription unit encoding subtype H7 hemagglutinin.

Figure 4B is a schematic representation of the nonretroviral vector comprising the influenza virus antigen DNA transcription unit encoding subtype H1 hemagglutinin.

Figure 4C is a schematic representation of the nonretroviral vector comprising a control DNA transcription unit, encoding no influenza virus antigens.

Figure 5 is a bar graph depicting the maximum median weight loss for DNA-vaccinated mice in experiment 4, Table 7.

Detailed Description of the Invention

This invention relates to a method of immunizing vertebrates, particularly mammals, including humans, against a pathogen, or infectious agent, thereby eliciting humoral and/or cell-mediated immune responses which limit the spread or growth of the infectious agent and result in

-4-

protection against subsequent challenge by the pathogen or infectious agent.

The term "immunizing" refers herein to the production of an immune response in a vertebrate which protects (partially or totally) from the manifestations of infection (i.e., disease) caused by an infectious agent. That is, a vertebrate immunized by the present invention will not be infected or will be infected to a lesser extent than would occur without immunization.

10 A DNA transcription unit is a polynucleotide sequence which includes at least two components: antigen-encoding DNA and transcriptional promoter elements. A DNA transcription unit may optionally include additional sequences, such as: enhancer elements, splicing signals, 15 termination and polyadenylation signals, viral replicons and bacterial plasmid sequences.

The DNA transcription unit can be produced by a number of known methods. For example, using known methods, DNA encoding the desired antigen can be inserted 20 into an expression vector to construct the DNA transcription unit. See Maniatis et al., Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989).

The DNA transcription unit can be administered to an 25 individual, or inoculated, in the presence of adjuvants or other substances that have the capability of promoting DNA uptake or recruiting immune system cells to the site of the inoculation. It should be understood that the DNA transcription unit itself will be expressed by host cell 30 factors.

The "desired antigen" can be any antigen expressed by an infectious agent or any antigen that has been determined to be capable of eliciting a protective response against an infectious agent. These antigens may 35 or may not be structural components of the infectious

-5-

agent. The encoded antigens can be translation products or polypeptides. The polypeptides can be of various lengths. They can undergo normal host cell modifications such as glycosylation, myristoylation or phosphorylation. 5 In addition, they can be designed to undergo intracellular, extracellular or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells.

Potential pathogens for which the DNA transcription unit can be used include DNA encoding antigens derived 10 from any virus, chlamydia, mycoplasma, bacteria, parasite or fungi. Viruses include the herpesviruses, orthomyxoviruses, rhinoviruses, picornaviruses, adenoviruses, paramyxoviruses, coronaviruses, 15 rhabdoviruses, togaviruses, flaviviruses, bunyaviruses, rubella virus, reovirus, hepadna viruses and retroviruses including human immunodeficiency virus. Bacteria include mycobacteria, spirochetes, rickettsias, chlamydia, and mycoplasma. Fungi include yeasts and molds. Parasites 20 include malaria. It is to be understood that this list does not include all potential pathogens against which a protective immune response can be generated according to the methods herein described.

An individual can be inoculated through any 25 parenteral route. For example, an individual can be inoculated by intranasal, intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular methods. In a particular embodiment of the present invention, an individual is vaccinated by contacting a mucosal surface 30 on the individual with the desired DNA transcription unit in a physiologically compatible medium. The DNA transcription unit can be administered to a mucosal surface by a variety of methods, including DNA-containing nose-drops, inhalants and suppositories.

-6-

Any appropriate physiologically compatible medium, such as saline, is suitable for introducing the DNA transcription unit into an individual.

The following Examples describe vaccination trials using direct DNA inoculations designed for use in both avian and murine influenza virus models. Both of these models afford rapid assays for protective immunizations against lethal challenges, wherein challenge of an unimmunized animal causes death within 1-2 weeks.

Immunization as described herein has been accomplished with DNA transcription units (i.e., vectors) that express an influenza virus hemagglutinin glycoprotein. This protein mediates adsorption and penetration of virus and is a major target for neutralizing antibodies. Influenza virus hemagglutinin proteins have 14 different serological subtypes. In the avian model, DNA expression vectors for the H7 subtype (comprising a DNA transcription unit encoding the H7 subtype hemagglutinin) have been used to provide protection against challenge with an H7N7 virus. In the murine model, a DNA transcription unit expressing the H1 hemagglutinin was used to immunize against an H1N1 virus.

Example 1 - Immunization of Chickens Against Influenza Virus

25 Procedure:

A DNA transcription unit referred to as pP1/H7 (Fig. 1), encoding a replication competent avian leukosis virus expressing the influenza virus hemagglutinin type 7 (H7) gene was constructed as described in Hunt *et al.*, *J. of Virology*, 62(8):3014-3019 (1988). DNA unit p188 (Fig. 2) encoding a replication defective derivative of pP1/H7 that expresses H7 but is defective for the avian virus vector polymerase and envelope proteins was constructed by deleting an XbaI fragment from pP1/H7. DNA unit pRCAS



-7-

(Fig. 3), encoding the avian leukosis virus vector, with no influenza virus insert, was constructed as described in Hughes *et al.*, *J. of Virology*, 61:3004 (1987). DNA units were diluted in saline at a concentration of 100 µg per 0.2 ml for inoculation.

To test the ability of the inoculated DNA to protect against a lethal influenza virus challenge, groups of three-week old chicks were inoculated with pP1/H7, p188, or pRCAS DNA. Specific pathogen free chicks that are maintained as an avian-leukosis virus-free flock (SPAFAS, Norwich, CT) were used for inoculations. Each chick received 100 µg of DNA ( $\sim 1 \times 10^{13}$  molecules) intravenously (iv), 100 µg intraperitoneally (ip), and 100 µg subcutaneously (sc). Four weeks later chicks were bled and boosted with 300 µg of DNA (100 µg iv, 100 µg ip, and 100 µg sc). At one week post-boost, chicks were bled and challenged by the nares with 100 lethal doses ( $1 \times 10^4$  egg infectious doses) of a highly pathogenic type H7 avian influenza virus, A/Chicken/Victoria/1/85 (H7N7) (CK/Vic/85). The chickens were observed daily for ten days for signs of disease. One and one half weeks after challenge, sera were obtained from surviving birds. These as well as the pre- and post-boost sera were used for analyses for hemagglutination inhibiting antibodies (HI). Sera were analyzed in microtiter plates with receptor-destroying enzyme-treated sera as described by Palmer *et al.*, *Advanced Laboratory Techniques for Influenza Diagnosis*, p. 51-52, Immunology series no. 6, U.S. Department of Health, Education, and Welfare, Washington, D.C. (1975).

#### Results:

The H7-expressing DNA transcription units protected each of the chickens inoculated with pP1/H7 or p188 (Table 1). In contrast, inoculation with the control DNA, pRCAS,

-8-

failed to protect the chickens against lethal virus challenge. The birds in the control group started to show signs of disease on the second day post-challenge. By the third day, three of the six control birds had died and all control birds were dead by the fifth day. The birds inoculated with hemagglutinin-expressing DNAs showed no signs of disease. By one and one half weeks post challenge both of these groups had developed high levels of HI antibody.

10 Example 2 - Immunization Against Influenza Virus is  
Reproducible

To assess the reproducibility of the protection elicited by immunization with the replication-defective H7-expressing DNA, the experiment described in Example 1 was repeated three times using only p188 and pRCAS DNAs for inoculations. The results of the repeat experiments confirmed that the H7-expressing p188 DNA could afford protection against a lethal challenge (Table 2). In contrast to the first experiment, in which all of the p188-inoculated chickens survived the lethal challenge, immunizations in the 2nd, 3rd, and 4th experiments achieved only partial protection with from 28% to 83% of the vaccinated birds surviving. Further, in contrast to the first experiment in which vaccinated birds showed no signs of disease, most of the survivors of the repeat experiments showed transient signs of post-challenge sickness. As in the first experiment, the control DNA did not provide protection. Summing the results of the 4 experiments, 28 out of 56 p188-vaccinated birds survived whereas only 1 of 55 control DNA-inoculated birds survived. Thus, despite the variability, significant immunization was achieved.

-9-

Example 3 - Immunization can be Accomplished by Several  
Different Routes of Inoculation

Procedure:

The DNA encoding p188-H7 and control DNA were tested  
5 again for the ability to protect against a lethal  
influenza virus challenge. This experiment included a  
group that was vaccinated and boosted by three routes of  
inoculation (i.e., ip, iv and sc), a group that was  
vaccinated by the same three routes but did not receive a  
10 boost, small groups that were vaccinated and boosted by  
only one route of inoculation and a control group treated  
with the anti-influenza virus drug, amantadine-HCL. This  
last group was included to allow the comparison of  
antibody responses to the challenge virus in vaccinated  
15 and unvaccinated chickens. The amantadine-treated birds  
were given 0.01% amantadine in their drinking water  
beginning 8 hours after challenge and were also injected  
ip with 1.0 ml of 0.1% amantadine 24 and 48 hours after  
challenge.

20 Results:

The results of this experiment confirmed that the  
replication defective H7-expressing DNA (p188) could  
afford protection against a lethal virus challenge  
(Table 3). The p188 immunized birds showed transient  
25 signs of sickness following the challenge. As in the  
previous experiments, the control DNA did not provide  
protection. All of the 5 amantadine-treated control birds  
developed disease. Four of these survived the challenge,  
providing sera that could be used to compare the time  
30 course and specificity of anti-influenza virus responses  
in immunized and non-immunized chickens (see Example 5  
below).

-10-

Example 4 - Immunization can be Accomplished by Several  
Different Routes of Inoculation

Procedure:

A third experiment was initiated to increase the  
5 numbers of birds in the test groups and to further  
evaluate the efficacy of different routes of immunization.  
In this experiment 12 chicks were inoculated with 100 µg  
p188 by the iv, ip, and sc routes, 8 chicks were  
inoculated iv-only and 8 ip-only. For controls, 12 chicks  
10 were inoculated with pRCAS and 12 chicks were not  
inoculated. All immunizations were followed by a boost  
four weeks after the initial inoculation. The boosts used  
the same DNA dose and sites of inoculation as the  
vaccinations. The control and immunized animals were  
15 challenged with ck/vic/85 1-2 weeks after the boost, with  
high challenge doses used in order to achieve essentially  
100% killing within 1-2 weeks.

Results:

The results again demonstrated protection by p188  
20 (Table 4). Eight of the 12 p188 immunized birds survived,  
whereas all 12 of the control pRCAS chickens died. The  
twelve birds in the untreated control group also had no  
survivors. Six out of the 8 chickens inoculated iv-only  
with p188 survived whereas none of the 8 chickens  
25 inoculated ip-only survived.

Example 5 - Analysis of Antibody Response to Challenge  
Virus in Vaccinated and Unvaccinated Animals

Procedure:

To allow the comparison of antibody responses to the  
30 challenge virus in vaccinated and unvaccinated chickens,  
experiment 2 from Example 2 (Table 2) included a non-  
vaccinated group rescued with the anti-influenza A virus  
drug, amantadine-HCL (Table 2) (Webster, R.G., et al., J.

-11-

Virol. 55:173-176 (1985)). All of the 5 amantadine-treated birds developed disease. Four of these survived, providing sera that could be used to compare antibody responses in immunized and non-immunized chickens

5 (Table 6).

Sera from p188 inoculated and amantadine treated birds in the second experiment were analyzed for the time course of antibody responses to H7 and to other influenza virus proteins (Table 6). Antibody responses to H7 were  
10 quantitated using hemagglutination inhibition as well as virus neutralization and enzyme-linked immunosorbent assays (ELISA) for antibody. Neutralizing antibody was determined in chick embryo fibroblast cultures with 200 TCID<sub>50</sub> of virus using cytopathology and hemagglutinin for  
15 detection of virus replication.

#### Results:

Analysis of the antibody responses in vaccinated and amantadine-rescued birds revealed that the p188-inoculations had primed an antibody response to H7  
20 (Table 6). As in experiment 1 (Table 1), DNA vaccination and boost induced only low titers of antibody to H7. However, within one week of challenge, the DNA-immunized group had high titers of HI and neutralizing activity for H7. These titers underwent little (if any) increase over  
25 the next week. Furthermore, most of the post-challenge antibody in the vaccinated birds was directed against H7. This specificity was shown by comparing ELISA antibody titers to H7 virus (the immunizing hemagglutinin type) and H5 virus (a hemagglutinin type to which the birds had not  
30 been exposed). The post-challenge sera contained 20-times higher titers of ELISA antibody for the H7 than the H5 virus (Table 6). By contrast, in the amantadine-rescued group, antibodies did not appear until two weeks post-challenge. Most of this response was not H7-specific.

-12-

This was demonstrated by the post-challenge sera from the amantadine-rescued birds which had comparable titers of ELISA antibody for the H5 and the H7 influenza viruses. (Table 6).

5 Example 6 - Immunization of Chickens and Mice Using a  
Nonretroviral Transcription Unit

Procedure

This experiment was performed in order to demonstrate that DNA transcription units devoid of  
10 retroviral DNA could be successfully employed to generate a protective immune response in both chickens and mice according to the methods herein described. The vectors used in this experiment to vaccinate chicken and mice are shown in Figure 4A-4C. Figure 4A  
15 is a schematic representation of pCMV-H7, a plasmid capable of expressing the influenza virus H7 subtype hemagglutinin under the transcription control of a cytomegalovirus (CMV) immediate early promoter. Figure 4B is a schematic showing pCMV-H1, a plasmid capable of  
20 expressing the influenza virus H1 subtype hemagglutinin under the control of a CMV immediate early promoter. This is the DNA transcription unit used in the mouse experiments. Figure 4C shows pCMV, a control plasmid which is not capable of expressing influenza antigens.  
25 These plasmids are derivatives of the pBC12/CMV vector of Dr. Brian Cullen, Duke University, Durham, North Carolina.

In the chicken and mouse experiments using pCMV-H7 and pCMV-H1 DNAs (the nonretroviral-based DNA  
30 transcription units) to generate immune responses, 100 µg of DNA was inoculated intravenously, intraperitoneally, and intramuscularly. All vaccinations were followed by a boost 4 weeks later. The boosts used the same DNA dose and sites of

-13-

inoculation as the vaccinations. Challenge was 1-2 weeks after the boost, with high challenge doses being used so as to achieve essentially 100% killing within 1-2 weeks.

5 Results:

In five chicken trials using a nonretrovirus-based vector for vaccination (pCMV-H7) (Figure 4A), approximately 60% of the chickens were protected. In one mouse trial, six out of six vaccinated mice and  
10 only one out of six control mice survived. Thus, considerable protection has been achieved using nonretroviral DNA expression vectors (containing DNA transcription units encoding viral antigens) to  
15 vaccinate animals. See, for example, Table 5.

In the chicken experiments, protective responses were associated with the rapid appearance of H7-specific antibodies after challenge (Robinson et al., 1993). Sera contained low to undetectable levels of anti-H7 antibodies after vaccination and boost. The  
20 first mouse experiment was similar to the chicken experiments in that inoculated mice also had low titers of anti-hemagglutinin activity before challenge. However, as in the chicken experiments, high titers of antibody appeared after challenge. The vast majority  
25 of this antibody was IgG.

Example 7 - Immunization of Mice by Vaccination with a Nonretroviral Transcription Unit: Analysis of Various Routes of Inoculation

Procedure:

30 A DNA transcription unit referred to as pCMV-H1 (described in Figure 4B) was successfully used to immunize mice against a lethal challenge with mouse adapted A/PR/8/34 H1N1 influenza virus. This

-14-

transcription unit encodes an influenza type H1 hemagglutinin under the transcription regulation of a CMV immediate early promoter. The H1 influenza virus hemagglutinin gene used in this construct is described  
5 in more detail in Winters et al., Nature 292:72 (1981).

The first experiment was conducted by inoculation of 6-8 week old Balb/C mice with 100  $\mu$ g of pCMV-H1 DNA by each of three routes; iv, ip and im. The second, third and fourth experiments each included one group of  
10 mice inoculated iv, ip and im, as well as additional groups representing different routes of inoculation (data summarized in Table 7 and Figure 5.

The numbers in Table 7 represent the number of surviving mice/number of inoculated mice. The routes  
15 of inoculation (iv, intravenous; ip, intraperitoneal; im, intramuscular; sc., subcutaneous; in, intranasal; id, intradermal) for each trial are indicated. In most instances, 100  $\mu$ g of DNA was administered per injection. Intramuscular (im) inoculations were given  
20 by injection of 100  $\mu$ g DNA in each hip muscle. Intravenous (iv) inoculations were given by injection in the tail vein. Intranasal (in) administrations of DNA and challenges were done on Metofane-anesthetized animals (Pitman-Moore) (these animals inhale deeply).  
25 Intradermal (id) inoculations were done in the foot pad using only 50  $\mu$ g of DNA. The control groups in experiments 2 and 3 received saline. The controls for experiment 1 received control DNA (vector without an insert encoding the antigen) administered iv, ip and  
30 im. The control group in experiment 4 received control DNA im, in and id. Occasional mice are resistant to influenza challenge. One of the survivors in the intranasal group in experiment 2, the one survivor in the control group in experiment 1, and 1 survivor in  
35 the control group in experiment 4 were such resistant



-15-

mice. All groups showed signs of sickness following challenge. Data on weight loss were also collected and are presented in Figure 5. The weight loss data provides a quantitative measure for the degree of sickness in the different experimental groups.

### Results:

The survival data, weight loss data and initial serology data from this series of experiments indicate that many routes of inoculation can provide protective immunity. In addition, these data demonstrate that intranasal inoculation (DNA nose drops administered to Metofane-anesthetized mice) can provide protective immunity to a lethal virus challenge. The method herein described may, therefore, provide means of stimulating mucosal immunity. (Table 7 and Figure 5]. Finally, these data demonstrate that some routes of inoculation are more effective than others for generating a protective immune response (Table 8).

### Example 8 - Antibody Responses to Challenge Virus in Animals Vaccinated with a Nonretroviral DNA Transcription Unit

Experiments analyzing the serum response in pCMV-H7-vaccinated chickens were performed as described in Example 4. pCMV-H7 immunizations primed antibody responses, with high titers of antibody to H7 appearing post-challenge (Table 9).

-16-

TABLE 1 - Protection Against Lethal H7N7 Influenza  
Virus with DNA Coding for H7 Hemagglutinin

| Group  | Sick/Dead/Total | HI TITERS                   |                          |                                 |
|--------|-----------------|-----------------------------|--------------------------|---------------------------------|
|        |                 | Post-<br>vaccine<br>4 weeks | Post-<br>boost<br>1 week | Post-<br>Challenge<br>1.5 weeks |
| pP1/H7 | 0/0/6           | <. <sup>a</sup>             | <.                       | 864<br>(160-1280)               |
| p188   | 0/0/6           | < <sup>b</sup>              | <                        | 427<br>(160-1280)               |
| PRCAS  | 6/6/6           | <                           | <                        | +                               |

<sup>a</sup> (<.) means one of six birds had an HI titer of 10.

<sup>b</sup> (<) means that all birds had titers of less than 10.

<sup>c</sup> (+) means that all birds died.

-17-

TABLE 2 - Reproducibility of Protection Against a Lethal H7 Virus Challenge by Immunization with an H7-expressing DNA<sup>a</sup>

Fate of challenge group (number of survivors/number tested)

| Experiment | p188 DNA | PRCAS DNA | Amantadine | No treatment |
|------------|----------|-----------|------------|--------------|
| 1          | 6/6      | 0/6       | -          | -            |
| 2          | 5/6      | 1/5       | 4/5        | -            |
| 3          | 9/32     | 0/32      | -          | -            |
| 4          | 8/12     | 0/12      | -          | 0/12         |
| Total      | 28/56    | 1/55      | 4/5        | 0/12         |

<sup>a</sup> Experiment 1 is the same as that presented in Table 1. Challenge was at one week post boost in experiment 1 and at two weeks post boost in experiments 2, 3 and 4, -, not tested.

Three-week-old SPAFAS chicks were inoculated with 100 µg of DNA by each of three routes (iv, ip and sc). Four weeks later, they were boosted by inoculation with 100 µg of DNA administered iv, ip and sc. One to two weeks later, chickens were challenged via the nares with 100 lethal doses of A/Ck/Vic/85 (H7N7). Some survivors suffered transient signs of influenza virus infections.

-18-

TABLE 3 - Protection Against Lethal H7N7 Influenza Virus  
with DNA Coding for H7 Hemagglutinin

| Group                      | Route of Inoculation | Boost | Sick/Dead/Total <sup>a</sup> |
|----------------------------|----------------------|-------|------------------------------|
| p188                       | ip/iv/sc             | yes   | 6/1/6                        |
| p188                       | iv only              | yes   | 1/1/2                        |
| p188                       | ip only              | yes   | 0/0/2                        |
| p188                       | sc only              | yes   | 2/2/2                        |
| PRCAS                      | ip/iv/sc             | yes   | 5/4/5                        |
| none                       | NA <sup>b</sup>      | NA    |                              |
| none<br>Aman. <sup>c</sup> | NA                   | NA    | 5/1/5                        |
| p188                       | iv/ip/sc             | no    | 4/4/6                        |
| PRCAS                      | iv/ip/sc             | no    | 6/6/6                        |

<sup>a</sup> Sick birds that survived developed only mild signs of sickness such as ruffled feathers and temporary loss of appetite.

<sup>b</sup> (NA) not applicable.

<sup>c</sup> (Aman.) is an abbreviation for Amantadine.

-19-

TABLE 4 - Protection Against Lethal H7N7 Influenza Virus with DNA Coding for H7 Hemagglutinin

| Group | Route of Inoculation | Boost | Sick/Dead/Total <sup>a</sup> |
|-------|----------------------|-------|------------------------------|
| p188  | iv/ip/sc             | yes   | 6/4/12                       |
| p188  | iv only              | yes   | 2/2/8                        |
| p188  | ip only              | yes   | 8/8/8                        |
| PRCAS | iv/ip/sc             | yes   | 12/12/12                     |
| none  | NA <sup>b</sup>      | NA    | 12/12/12                     |

<sup>a</sup> Sick birds that survived developed only mild signs of sickness such as ruffled feathers and temporary loss of appetite.

<sup>b</sup> (NA) not applicable

-20-

TABLE 5 - Protection Against a Lethal H7 Influenza Virus  
Challenge by Immunization with pCMV-H7 DNA.

---

| Fate of challenge group (number<br>of survivors/number tested) |             |          |
|--|-------------|----------|
| <hr/>  |             |          |
| Trial  | pCMV-H7 DNA | pCMV DNA |
| 1  | 5/6         | 0/6      |
| 2  | 4/6         | 0/6      |
| 3  | 2/6         | 0/7      |
| 4  | 4/6         | 1/7      |
| 5  | 4/6         | 0/7      |
| Total  | 19/30       | 1/33     |

---

Immunization and boosts were the same as in Table 2.  
Some survivors developed transient signs of influenza-  
related illness.

-21-

TABLE 6- Antibody Response in H7-Immunized and Amantadine-Treated Birds

| Grp.         | No. <sup>a</sup> | Bleed                | HI             | Antibody to<br>Ck/Vic/85<br>(H7N7) |                            | Antibody to<br>Ck/Penn/1370/83<br>(H5N2) |                            |
|--------------|------------------|----------------------|----------------|------------------------------------|----------------------------|--|----------------------------|
|              |                  |                      |                | Neutralizing                       | ELISA ( $\times 10^{-3}$ ) | ELISA ( $\times 10^{-3}$ )               | ELISA ( $\times 10^{-3}$ ) |
| p188         | 6                | 1 wk PB <sup>b</sup> | 5 (0-10)       | 2 (0-10)                           | 2 (0-10)                   | <  | <                          |
|              | 6                | 2 wk PB              | 8 (0-20)       | 13 (0-33)                          | 5 (0-10)                   | <  | <                          |
|              | 5                | 1 wk PC <sup>c</sup> | 112 (80-160)   | 873 (33-3333)                      | 640 (100-1000)             | 26 (0-100)                               | 26 (0-100)                 |
|              | 5                | 2 wk PC              | 272 (80-640)   | 540 (33-1000)                      | 640 (100-1000)             | 46                                       | 46                         |
| None<br>Aman | 5                | 1 wk PB              | < <sup>d</sup> |                                    | <                          | <  | <                          |
|              | 5                | 2 wk PB              | <              | <                                  | <                          | <  | <                          |
|              | 4                | 1 wk PC              | <              | <                                  | <                          | <  | <                          |
|              | 4                | 2 wk PC              | 300 (80-640)   | 442 (100-1000)                     | 1000 (1000)                | 1000 (1000)                              | 1000 (1000)                |

Antibody titers are given as the median (range).

<sup>a</sup> (No.) Number of chicks in group at time of bleed.<sup>b</sup> (wk PB) means weeks post boost.<sup>c</sup> (wk PC) means weeks post challenge.<sup>d</sup> (<) means all birds had titers of less than 10.

-22-

TABLE 7 - Survival Data for Four DNA Immunization Trials Using pCMV-H1  
in the Murine/Influenza Virus Model

| Trial | Control | iv,ip, |       | in    | iv    | id   | sc  | ip  |
|-------|---------|--------|-------|-------|-------|------|-----|-----|
|       |         | im,    | im    |       |       |      |     |     |
| exp 1 | 1/6     | 6/6    |       |       |       |      |     |     |
| exp 2 | 0/6     | 6/6    | 5/6   | 6/6   | 4/6   |      | 4/6 | 0/6 |
| exp 3 | 0/6     | 6/6    | 6/6   | 3/6   | 6/6   | 6/6  |     |     |
| exp 4 | 2/6     | 3/4    | 7/7   | 4/5   |       | 3/6  |     |     |
| Total | 3/24    | 21/22  | 18/19 | 13/17 | 10/12 | 9/12 | 4/6 | 0/6 |



-23-

TABLE 8 - HI Antibody Titers Following Inoculation of PCMV-H1

| Time of bleed           | Trial | Control | iv, ip, im | in  | iv  | id  | sc  |
|-------------------------|-------|---------|------------|-----|-----|-----|-----|
| Prebleed                | 1     | <       | <          | <   | <   | <   | <   |
|                         | 2     | <       | <          | <   | <   | <   | <   |
|                         | 3     | <       | <          | <   | <   | <   | <   |
|                         | 4     | <       | <          | <   | <   | <   | <   |
| 4 wk pv (preboost)      | 1     | <       | <          | <   | <   | <   | <   |
|                         | 2     | <       | <          | <   | <   | <   | <   |
|                         | 3     | <       | 40         | <   | <   | <   | <   |
|                         | 4     | <       | <          | <   | <   | <   | <   |
| 10 da PB (prechallenge) | 1     | <       | <          | <   | <   | <   | <   |
|                         | 2     | <       | 40         | <   | <   | <   | <   |
|                         | 3     | <       | 80         | <   | 40  | <   | <   |
|                         | 4     | <       | <          | <   | <   | <   | <   |
| 4-5 da PC               | 1     | <       | <          | <   | <   | <   | <   |
|                         | 2     | <       | <          | <   | <   | <   | <   |
|                         | 3     | <       | 80         | <   | 80  | <   | <   |
|                         | 4     | <       | <          | <   | <   | <   | <   |
| 14-19 da PC             | 1     | d*      | 2560       | 320 | 320 | 640 | 640 |
|                         | 2     | d       | 640        | 320 | 320 | 640 | 640 |
|                         | 3     | d       | 160        | 320 | 640 | 640 | 640 |
|                         | 4     | d**     | 640        | 640 | 640 | 640 | 640 |

Serology for trials reported in Table 7. Data is for pooled sera. Designations and titers are the same as those in Table 9 with the exception of: control; da, days. \*One surviving mouse had a titer of 80. \*\*Two surviving mice had titers of 320.

-24-

TABLE 9 - Antibody Responses to the H7 Challenge Virus in pCMV-H7 and pCMV-control DNA inoculated chickens

| Time of bleed                  | Trial | Control-DNA-inoculated |                   |                               | CMV-H7-DNA-inoculated |                   |                               |
|--------------------------------|-------|------------------------|-------------------|-------------------------------|-----------------------|-------------------|-------------------------------|
|                                |       | HI                     | Neut-<br>ralizing | ELISA<br>(x10 <sup>-3</sup> ) | HI                    | Neut-<br>ralizing | ELISA<br>(x10 <sup>-3</sup> ) |
| 4 wk PV<br>(preboost)          | 2     | <                      | <                 | <                             | <                     | <                 | <                             |
|                                | 3     | <                      | <                 | <                             | <                     | <                 | <                             |
|                                | 4     | <                      | <                 | <                             | <                     | <                 | <                             |
|                                | 5     | <                      | <                 | <                             | 2.5                   | <                 | <                             |
| 1 wk PB<br>(pre-<br>challenge) | 2     | <                      | <                 | <                             | <                     | <                 | <                             |
|                                | 3     | <                      | <                 | <                             | <                     | <                 | <                             |
|                                | 4     | <                      | <                 | <                             | 2.5                   | <                 | 2.5                           |
|                                | 5     | <                      | <                 | <                             | 2.5                   | <                 | 2.5                           |
| 2 wk PC                        | 2     | D                      | D                 | D                             | 60                    | 33                | 765                           |
|                                | 3     | D                      | D                 | D                             | 60                    | 33                | 1000                          |
|                                | 4     | D*                     | D*                | D*                            | 100                   | 33                | 775                           |
|                                | 5     | D                      | D                 | D                             | 140                   | 108               | 1000                          |

Designations and titers are as in Table 3 except for: PV, post vaccination and D, dead.  
 \*One control bird survived in this group. Its post challenge titers were HI, 80; Neutralizing antibody, 10; and ELISA, 100. Control birds did not receive DNA.

-25-

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

-26-

CLAIMS

1. A product for use in vertebrate therapy, e.g., immunization, contraception or tumor therapy, and comprising a DNA transcription unit comprising DNA encoding a desired therapeutic agent operatively linked to a promoter region.
2. Use of a DNA transcription unit comprising DNA encoding a desired antigen operatively linked to a promoter region, for the manufacture of a medicament for use in vertebrate immunization by eliciting a humoral immune response, a cell-mediated immune response or both against the desired antigen.
3. A method of immunizing a vertebrate, said method comprising administering to a vertebrate a DNA transcription unit comprising DNA encoding a desired antigen operatively linked to a promoter region, whereby a humoral immune response, a cell-mediated immune response or both is elicited against the desired antigen.
4. Use according to Claim 2 or a method according to Claim 3 wherein the desired antigen is capable of eliciting a protective immune response against an infectious agent.
5. Use according to Claim 2 or Claim 4, wherein the medicament comprises a physiologically acceptable carrier and is adapted to be administered by a route chosen from mucosal, intranasal, intravenous, intramuscular, intraperitoneal, intradermal and subcutaneous.

-27-

6. The method of Claim 3 or Claim 4, wherein the DNA transcription unit, in a physiologically acceptable carrier, is administered to a vertebrate through a route of administration chosen from intranasal,  
5 intravenous, intramuscular, intraperitoneal, intradermal and subcutaneous.
7. The method of Claim 3 or Claim 4, wherein the DNA transcription unit is administered to a vertebrate by contacting the DNA transcription unit in a  
10 physiologically acceptable carrier with a mucosal surface of the vertebrate.
8. A method of immunizing a vertebrate against an infectious agent, said method comprising administering to a mucosal (e.g., nasal) surface of a  
15 vertebrate a DNA transcription unit comprising DNA encoding a desired antigen operatively linked to a promoter region, in a physiologically acceptable carrier, thereby eliciting a humoral or cell-mediated immune response, or both, against the desired  
20 antigen, whereby the vertebrate is protected from disease caused by an infectious agent.
9. A product, use or method according to any one of the preceding claims, wherein the DNA transcription unit is of nonretroviral origin.
- 25 10. Use or a method according to any one of Claims 2 to 9, wherein the antigen is viral.
11. Use or a method according to Claim 10, wherein the virus is an influenza virus, e.g., virus hemagglutinin.

-28-

12. A product, use or method according to any one of the preceding claims, wherein the vertebrate is a mammal, e.g., a human.

1/7

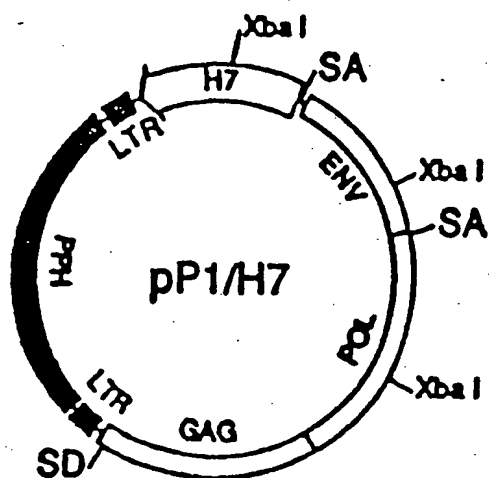


Figure 1.

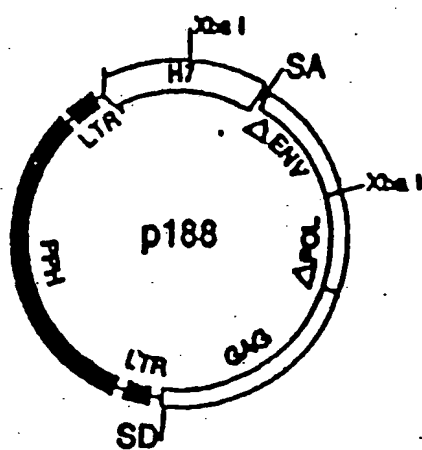


Figure 2.



3/7

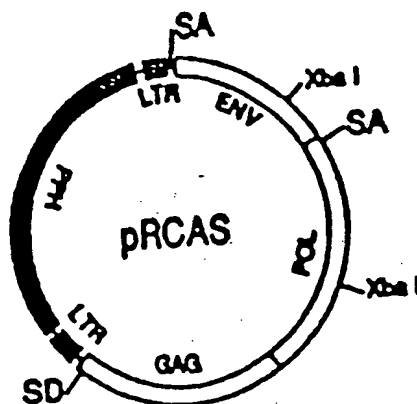


Figure 3.

4/7

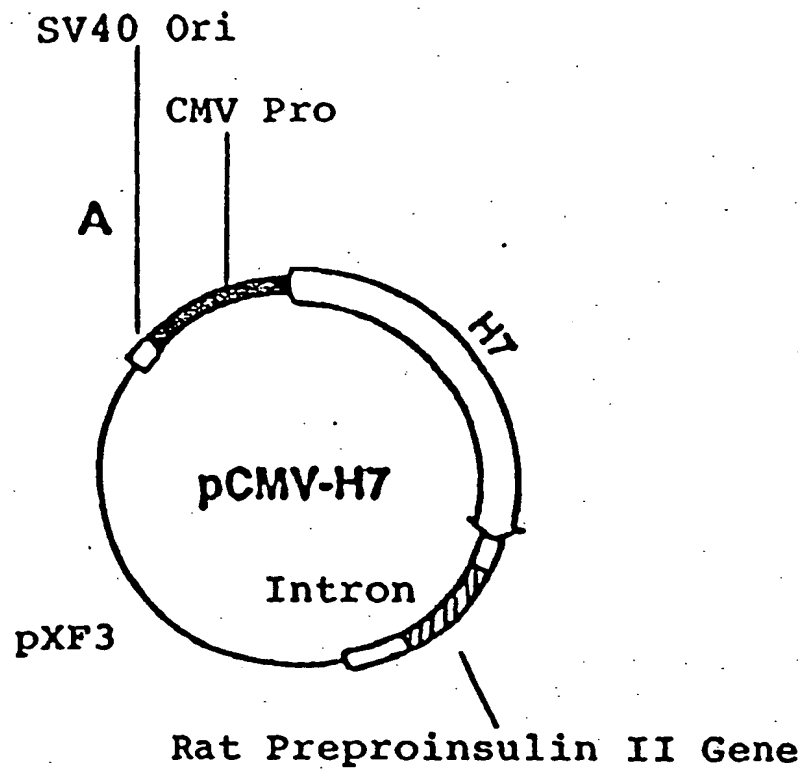


Figure 4A

5/7

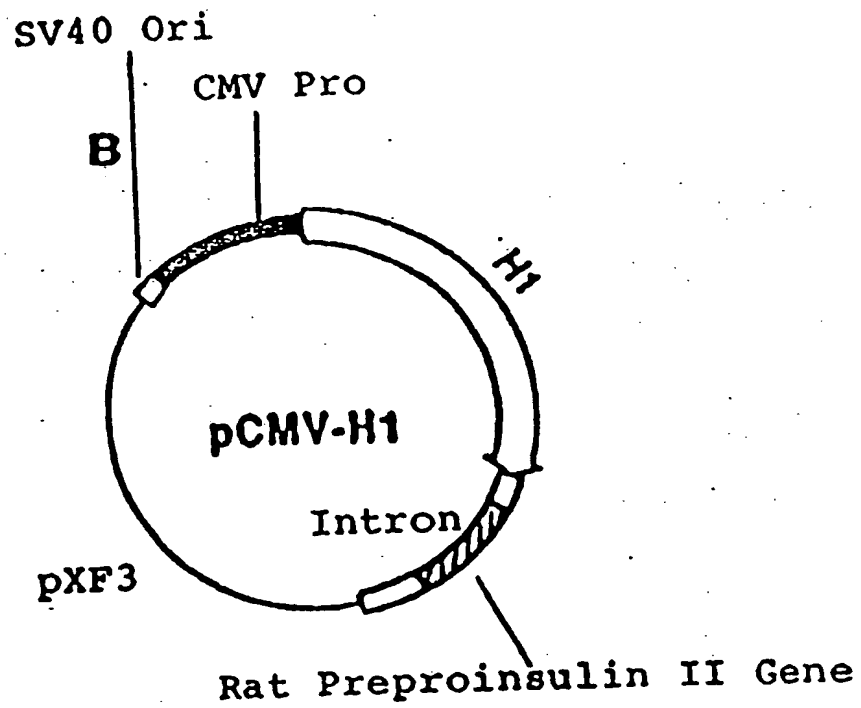


Figure 4B

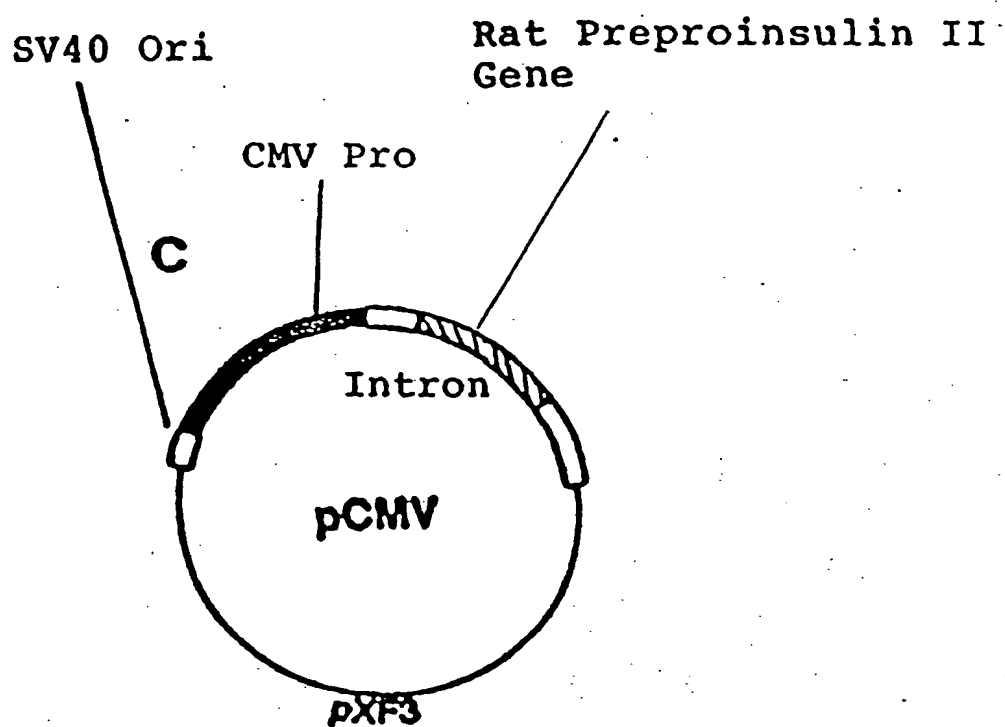


Figure 4C

7/7

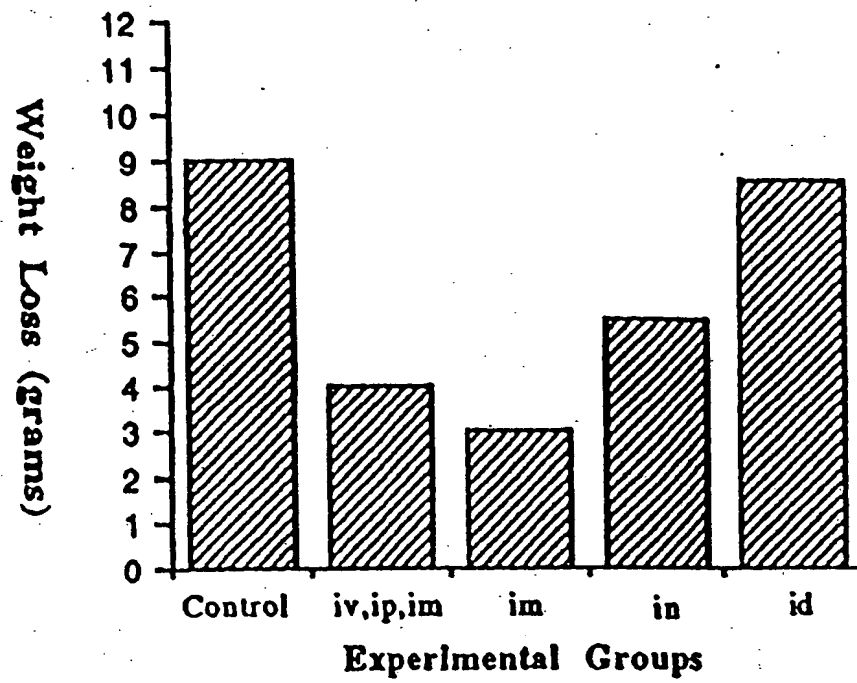


Figure 5

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/02394

|   |  |  |
|---|--|--|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>   |  |  |
| According to International Patent Classification (IPC) or to both National Classification and IPC<br>Int.Cl. 5 C12N15/44;                      C12N15/86;                      A61K39/145   |  |  |
| <b>II. FIELDS SEARCHED</b>  |  |  |
| Minimum Documentation Searched <sup>7</sup>   |  |  |
| Classification System   | Classification Symbols   |  |
| Int.Cl. 5   | C12N ;                      C07K   |  |
| Documentation Searched other than Minimum Documentation<br>to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>   |  |  |
|   |  |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>   |  |  |
| Category <sup>9</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup>  |
| X   | JOURNAL OF VIROLOGY<br>vol. 62, no. 8, August 1988,<br>pages 3014 - 3019<br>HUNT, L. A. ET AL. 'Retrovirus expressed<br>hemagglutinin protects against lethal<br>Influenza virus infections'<br>cited in the application<br>see the whole document | 1-8,<br>10-12  |
| X   | WO,A,9 011 092 (VICAL, INC. WISCONSIN<br>ALUMNI RESEARCH FOUNDATION)<br>4 October 1990<br>see the whole document   | 1-6,9,12   |
| X   | WO,A,8 600 930 (WORCESTER FOUNDATION FOR<br>EXPERIMENTAL BIOLOGY)<br>13 February 1986<br>see the whole document  | 1-6,<br>11-12  |
| -/-   |  |  |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> |  |  |
| <b>IV. CERTIFICATION</b>  |  |  |
| Date of the Actual Completion of the International Search<br><div style="text-align: center; font-weight: bold;">02 JULY 1993</div>   |  | Date of Mailing of this International Search Report<br><div style="text-align: center;">02 JUL 1993</div>    |
| International Searching Authority<br><div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>   |  | Signature of Authorized Officer<br><div style="text-align: center; font-weight: bold;">CHAMBONNET F.J.</div> |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |   |                       |
|--|---|-----------------------|
| Category *   | Citation of Document, with indication, where appropriate, of the relevant passages  | Relevant to Claim No. |
| X  | WO,A,8 607 593 (BIOTECHNOLOGY RESEARCH PARTNERS, LTD)<br>31 December 1986<br>see the whole document<br>---  | 1-7, 10               |
| X  | EP,A,0 292 879 (ORION CORPORATION LTD)<br>30 November 1988<br>see the whole document<br>---   | 1,9-12                |
| X  | WO,A,9 201 045 (EQUINE VIROLOGY RESEARCH FOUNDATION, UNIVERSITY OF GLASGOW)<br>23 January 1992<br>see the whole document<br>---   | 1-12                  |
| X  | WO,A,9 002 803 (INSTITUTE FOR ANIMAL HEALTH LTD, RHONE-MERIEUX SA)<br>22 March 1990<br>see the whole document<br>---  | 1-11                  |
| X  | US,A,4 722 848 (PAOLETTI, E. & PANICALI, D.)<br>2 February 1988<br>see the whole document<br>---  | 1-6,9-12              |
| X  | GB,A,2 166 349 (AMERICAN HOME PRODUCT CORPORATION)<br>8 May 1986<br>see the whole document<br>---   | 1-12                  |
| X  | WO,A,9 002 797 (NORTH CAROLINA STATE UNIVERSITY)<br>22 March 1990<br>see the whole document<br>---  | 1-6,9-11              |
| X  | JOURNAL OF VIROLOGY<br>vol. 64, no. 3, March 1990,<br>pages 1070 - 1078<br>COSSET, F.L., ET AL. 'A new Avian Leukosis Virus-based packaging cell line that uses two separate transcomplementing helper genome'<br>see the whole document<br>----- | 1                     |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 93/02394

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 3 and partially 4 to 12 as far as they concern in vivo method of treatment or vaccination against a disease are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9302394  
SA 71686

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 02/07/93

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9011092                              | 04-10-90            | AU-A- 5344190              | 22-10-90            |
|   |                     | EP-A- 0465529              | 15-01-92            |
|   |                     | JP-T- 4504125              | 23-07-92            |
| WO-A-8600930                              | 13-02-86            | EP-A- 0188574              | 30-07-86            |
| WO-A-8607593                              | 31-12-86            | US-A- 4631191              | 23-12-86            |
|   |                     | AU-A- 6125386              | 13-01-87            |
|   |                     | EP-A- 0229826              | 29-07-87            |
|   |                     | US-A- 4920213              | 24-04-90            |
| EP-A-0292879                              | 30-11-88            | AU-B- 604122               | 06-12-90            |
|   |                     | AU-A- 1651488              | 01-12-88            |
|   |                     | CA-A- 1300052              | 05-05-92            |
|   |                     | JP-A- 63304988             | 13-12-88            |
| WO-A-9201045                              | 23-01-92            | AU-A- 8212891              | 04-02-92            |
|   |                     | CA-A- 2086740              | 07-01-92            |
|   |                     | EP-A- 0538299              | 28-04-93            |
| WO-A-9002803                              | 22-03-90            | AU-B- 633272               | 28-01-93            |
|   |                     | AU-A- 4214289              | 02-04-90            |
|   |                     | AU-B- 629248               | 01-10-92            |
|   |                     | AU-A- 4325089              | 02-04-90            |
|   |                     | EP-A- 0434721              | 03-07-91            |
|   |                     | EP-A- 0434747              | 03-07-91            |
|   |                     | WO-A- 9002802              | 22-03-90            |
|   |                     | JP-T- 4501658              | 26-03-92            |
|   |                     | JP-T- 4502852              | 28-05-92            |
| US-A-4722848                              | 02-02-88            | US-A- 4603112              | 29-07-86            |
|   |                     | AU-B- 561816               | 21-05-87            |
|   |                     | AU-A- 9180682              | 30-06-83            |
|   |                     | EP-A, B 0083286            | 06-07-83            |
|   |                     | US-A- 5174993              | 29-12-92            |
| GB-A-2166349                              | 08-05-86            | AU-B- 576907               | 08-09-88            |
|   |                     | AU-A- 4884085              | 08-05-86            |
|   |                     | CA-A- 1263305              | 28-11-89            |
|   |                     | DE-A- 3586841              | 24-12-92            |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9302394  
SA 71686

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

02/07/93

Page 2

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| GB-A-2166349                              |                     | EP-A, B 0181117            | 14-05-86            |
|   |                     | JP-A- 61118326             | 05-06-86            |
|   |                     | US-A- 4920209              | 24-04-90            |
| -----                                     |                     |                            |                     |
| WO-A-9002797                              | 22-03-90            | AU-A- 4307589              | 02-04-90            |
| -----                                     |                     |                            |                     |

EPO FORM P007

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82